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(54) Title: OLIGONUCLEOTIDES HAVING CHIRAL PHOSPHORUS LINKAGES

(57) Abstract

Sequence-specific oligonucleotides are provided having substantially pure chiral Sp phosphorothioate, chiral Rp phosphorothioate, chiral Sp alkylphosphonate, chiral Rp alkylphosphonate, chiral Sp phosphoamidate, chiral Rp phosphoamidate, chiral Sp phosphotriester, and chiral Rp phosphotriester linkages. The novel oligonucleotides are prepared via a stereospecific SN_2 nucleophilic attack of a phosphodiester, phosphorothioate, phosphoramidate, phosphotriester or alkylphosphonate anion on the 3′ position of a xylonucleotide. The reaction proceeds via inversion at the 3′ position of the xylo reactant species, resulting in the incorporation of phosphodiester, phosphorothioate, phosphoramidate, phosphotriester or alkylphosphonate linked ribofuranosyl sugar moieties into the oligonucleotide. Alternatively, enzymatic methods are used to prepare oligonucleotides having chirally pure phophorus inter-sugar linkages. Therapeutic and diagnostic methods using the same are also provided.

OLIGONUCLEOTIDES HAVING CHIRAL PHOSPHORUS LINKAGES

CROSS REFERENCE TO RELATED APPLICATIONS:

Portions of this application may have been supported by National Institute of Health Grant No. GM45061.

This application is a continuation-in-part of Application Serial No. US91/00243, filed January 11, 1991, which is a continuation-in-part of Application Serial No. 463,358 filed January 11, 1990 and of Application Serial No. 566,977, filed August 13, 1990. The entire disclosures of both applications, which are assigned to the assignee of this invention, are incorporated herein by reference.

FIELD OF THE INVENTION:

This invention is directed to sequence-specific oligonucleotides having chiral phosphorus linkages and to a novel enzymatic and chemical synthesis of these and other oligonucleotides. The invention includes chiral alkylphosphonate, chiral phosphotriester, chiral phosphorothioates, and chiral phosphoramidate-linked oligonucleotides. The invention

different from that of the natural phosphodiester oligonucleotides. Some are generally more chemically or thermodynamically stable than the natural phosphodiester oligonucleotides. At least the phosphorothioates have oligonucleotide-RNA heteroduplexes that can serve as substrates for endogenous RNase H.

The phosphorothioate oligonucleotides, like the natural phosphodiester oligonucleotides, are soluble in aqueous media. In contrast, methylphosphonate, phosphotriester, and phosphoramidate oligonucleotides, which lack a charge on the phosphorus group, can penetrate cell membranes to a greater extent and, thus, facilitate cellular uptake. The internucleotide linkage in methylphosphonate oligonucleotides is more base-labile than that of the natural phosphodiester internucleotide linkage, while the internucleotide linkage of the phosphorothioate oligonucleotides is more stable than the natural phosphodiester oligonucleotide linkage.

The resistance of phosphorothioate oligonucleotides to nucleases has been demonstrated by their long half-life in the presence of various nucleases relative to natural phosphodiester oligonucleotides. This resistance to nucleolytic degradation *in vitro* also applies to *in vivo* degradation by endogenous nucleases. This *in vivo* stability has been attributed to the inability of 3'-5' plasma exonucleases to degrade such oligonucleotides. Phosphotriester and methylphosphonate oligonucleotides also are resistant to

mers on hybridization becomes even more complex as chain length increases.

Bryant, F.R. and Benkovic, S.J. (1979), *Biochemistry*, 18:2825 studied the effects of diesterase on the diastereomers of ATP. Published patent application PCT/US88/03634 discloses dimers and trimers of 2'-5'- linked diastereomeric adenosine units. Niewiarowski, W., Lesnikowski, Z.J., Wilk, A., Guga, P., Okruszek, A., Uznanski, B., and Stec, W. (1987), *Acta Biochimica Polonia*, 34:217, synthesized diastereomeric dimers of thymidine, as did Fujii, M., Ozaki, K., Sekine, M., and Hata, T. (1987), *Tetrahedron*, 43:3395.

Stec, W.J., Zon, G., and Uznanski, B. (1985), *J. Chromatography*, 326:263, have reported the synthesis of certain racemic mixtures of phosphorothioate or methyphosphonate oligonucleotides. However, they were only able to resolve the diastereomers of certain small oligomers having one or two diastereomeric phosphorus linkages.

In a preliminary report, J.W. Stec, *Oligonucleotides as antisense inhibitors of gene expression: Therapeutic implications*, meeting abstracts, June 18-21, 1989, noted that a non-sequence-specific thymidine homopolymer octomer -- i.e. a $(dT)_8$-mer, having "all-except-one" Rp configuration methylphosphonate linkages -- formed a thermodynamically more stable hybrid with a 15-mer deoxyadenosine homopolymer -- i.e. a $d(A)_{15}$-mer -- than did a similar thymidine homopolymer having "all-except-one" Sp configuration methylphosphonate linkages. The hybrid between the "all-except-one" Rp $(dT)_8$-mer and the

possible to synthesize by chemical means diastereomerically pure chains of the length necessary for antisense inhibition," see J. Goodchild (1990) *Bioconjugate Chemistry*, 1:165.

The use of enzymatic methods to synthesize oligonucleotides having chiral phosphorous linkages has also been investigated. Burgers, P.M.J. and Eckstein, F. (1979), *J. Biological Chemistry*, 254:6889; and Gupta, A., DeBrosse, C., and Benkovic, S.J. (1982) *J. Bio. Chem.*, 256:7689 enzymatically synthesized diastereomeric polydeoxyadenylic acid having phosphorothioate linkages. Brody, R.S. and Frey, P.S. (1981), *Biochemistry*, 20:1245; Eckstein, F. and Jovin, T.M. (1983), *Biochemistry*, 2:4546; Brody, R.S., Adler, S., Modrich, P., Stec, W.J., Leznikowski, Z.J., and Frey, P.A. (1982) *Biochemistry*, 21: 2570-2572; and Romaniuk, P.J. and Eckstein, F. (1982) *J. Biol. Chem.*, 257:7684-7688 all enzymatically synthesized poly TpA and poly ApT phosphorothioates while Burgers, P.M.J. and Eckstein, F. (1978) *Proc. Natl. Acad. Sci. USA*, 75: 4798-4800 enzymatically synthesized poly UpA phosphorothioates. Cruse, W.B.T., Salisbury, T., Brown, T., Cosstick, R. Eckstein, F., and Kennard, O. (1986), *J. Mol. Biol.*, 192:891, linked three diastereomeric Rp GpC phosphorothioate dimers via natural phosphodiester bonds into a hexamer. Most recently Ueda, T., Tohda, H., Chikazuni, N., Eckstein, R., and Watanabe, K. (1991) *Nucleic Acids Research*, 19:547, enzymatically synthesized RNA's having from several hundred to ten thousand nucleotides intermittently incorporating Rp diastereomeric phosphorothioate linkages.

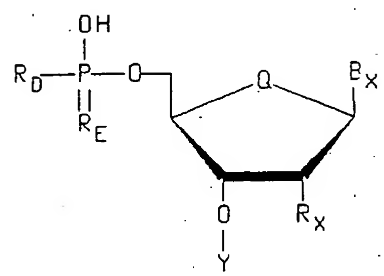
Another object is to provide therapeutic and research methods and materials for the treatment of diseases through modulation of the activity of DNA and RNA.

It is yet another object to provide new methods for synthesizing sequence-specific oligonucleotides having chirally pure phosphorothioate, methylphosphonate, phosphotriester or phosphoramidate linkages.

SUMMARY OF THE INVENTION:

The present invention provides stereoselective methods for preparing sequence-specific oligonucleotides having chiral phosphorous linkages. In certain preferred embodiments, these methods comprise the steps of:

(a) selecting a first synthon having structure (1):



(1)

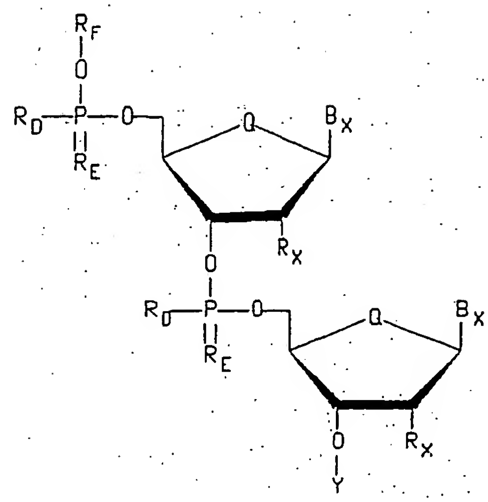
wherein:

Q is O or CH_2;

R_D is O, S, methyl, O-alkyl, S-alkyl, amino or substituted amino;

R_E is O or S;

R_X is H, OH, or a sugar derivatizing group;



(3)

via a stereospecific inversion of configuration at the 3′ position of the second synthon; and

(d) treating the new first synthon with a reagent to remove the labile blocking group R_F.

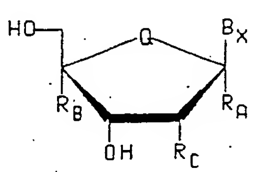
Additional nucleotides are added to the new first synthon by repeating steps (b), (c), and (d) for each additional nucleotide. Preferably, R_F is an acid-labile blocking group and said new first synthon in step (d) is treated with an acidic reagent to remove said acid-labile R_F blocking group.

The present invention also provides enzymatic methods for preparing oligonucleotides comprising nucleoside units joined together by either substantially all Sp or substantially all Rp phosphorus intersugar linkages comprising combining a sequence primer and a template and adding an excess of all four nucleoside triphosphates having a desired

substantially pure chiral phosphate linkages. In further embodiments, the oligonucleotides of the invention form at least a portion of a targeted RNA or DNA sequence.

The present invention also provides oligonucleotides comprising nucleoside units joined together by either all Sp phosphotriester linkages, all Rp phosphotriester linkages, all Sp phosphoramidate linkages, or all Rp phosphoramidate linkages. Also provided are oligonucleotides having at least 10 nucleoside units joined together by either all Sp alkylphosphonate linkages or all Rp alkylphosphonate linkages or all Rp phosphothioate linkages or all Sp phosphothioate* linkages. Preferably such alkylphosphonate linkages are methylphosphonate linkages. Each of these oligonucleotides can form at least a portion of a targeted RNA or DNA sequence.

In preferred embodiments of the invention, the oligonucleotides include non-naturally occurring nucleoside units incorporated into the oligonucleotide chain. Such nucleoside units preferably have structure (4) or structure (5):



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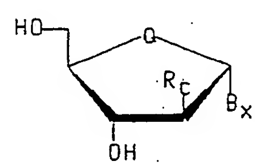
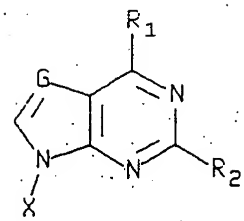


(5)

wherein Q is O or CHR_G, R_A and R_B are H, lower alkyl, substituted lower alkyl, an RNA cleaving moiety, a group which improves the pharmacodynamic properties of an oligonucleotide,



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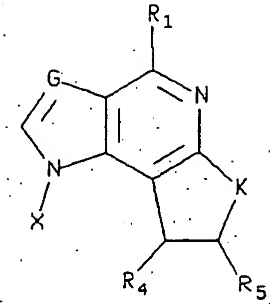

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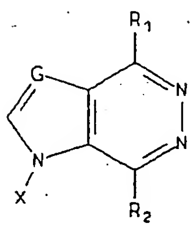

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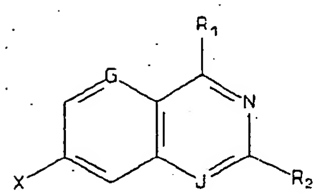

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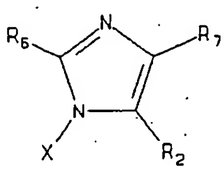

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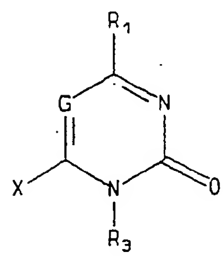


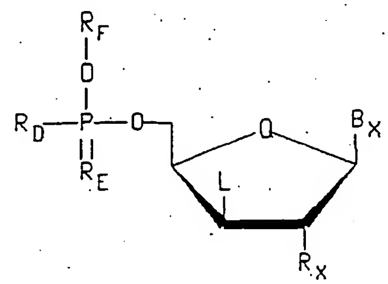
(11)

wherein:

G and K are, independently, C or N;

J is N or CR_2R_3;

The present invention also provides compounds which are useful in forming the oligonucleotides of invention. Such compounds have structure (12):



(12)

wherein Q is O or CH_2; R_D, R_E, R_X, L, and B_X are defined as above and R_F is H or a labile blocking group.

The oligonucleotides of the invention are useful to increase the thermodynamic stability of heteroduplexes with target RNA and DNA. Certain of the oligonucleotides of the invention are useful to elicit RNase H activity as a termination event. Certain other oligonucleotides are useful to increase nuclease resistance. The oligonucleotides of the invention are also useful to test for antisense activity using reporter genes in suitable assays and to test antisense activity against selected cellular target mRNA's in cultured cells. The production of protein may be modulated by contacting a cell with an oligonucleotide of the present invention wherein said oligonucleotide is complementary to at least a portion of a sequence of targeted RNA or DNA involved in the production of said protein.

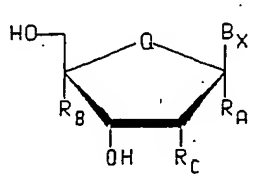
invention include nucleotides and oligonucleotides derived by replacement of one of the oxygen atoms of a naturally occurring phosphate moiety with a heteroatom, an alkyl group or an alkyoxy group. Thus, the terms "phosphate" or "phosphate anion" include naturally occurring nucleotides, phosphodiesters of naturally occurring oligonucleotides, as well as phosphorothioate, alkylphosphonate, phosphotriester, and phosphoamidate oligonucleotides.

Since there exist numerous phosphodiester linkages in an oligonucleotide, substitution of an oxygen atom by another atom such as, for example, sulfur, nitrogen, or carbon in one or more of the phosphate moieties yields a racemic mixture unless such substitution occurs in a stereospecific manner. As a practical matter, see Stec, W.J., Zon, G., and Uznanski, B. (1985), *J. Chromatography*, 326:263, above. Separation of the diastereomers of racemic mixtures of non-stereospecific synthesized oligonucleotides is only possible when there are a minimum of diasymmetric sites, for example, two diasymmetric sites. Since the diasymmetric substituent group at each diastereomeric phosphorus atom could have steric, ionic or other effects on conformation, binding, and the like at each such site, sequence-specific oligonucleotides having all Sp or all Rp chiral phosphorus linkages are desirable.

In accordance with this invention, sequence-specific oligonucleotides are provided comprising substantially pure chiral phosphate linkages such as, for example, phosphorothioate, methylphosphonate, phosphotriester or phosphoramidate

sequence. Thus, the present improvements are likely to lead to improved drugs, diagnostics, and research reagents.

Further improvements likely can be effected by making one or more substitutions or modifications to the base or the sugar moieties of the individual nucleosides employed to prepare the chiral oligonucleotides of the invention. Such substitutions or modifications generally comprise derivation at a site on the nucleoside base or at a site on the nucleoside sugar, provided such derivation does not interfere with the stereoselective syntheses of the present invention by, for example, blocking nucleophilic attack of the 5'-phosphate of a first synthon at the 3'-position of a second synthon. In certain embodiments, one or more of the nucleosides of the chiral oligonucleotides of the invention include a naturally occurring nucleoside unit which has been substituted or modified. These non-naturally occurring or "modified" nucleoside units preferably have either structure (4) or structure (5):



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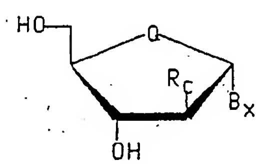


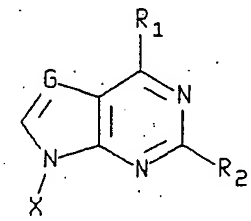
(5)

wherein:

Q is O or CHR_G;

R_A and R_B are H, lower alkyl, substituted lower alkyl, an RNA cleaving moiety, a group which improves the pharmacokinetic properties of an oligonucleotide, or a group

3,687,808 issued August 29, 1972. Preferably, B_x is selected such that a modified nucleoside has one of the structures (6)-(11):



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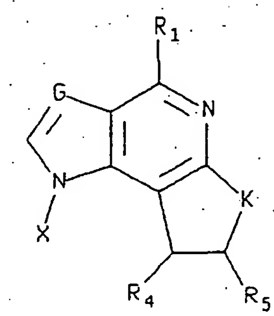

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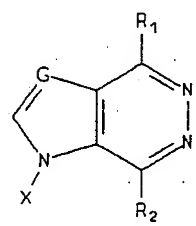

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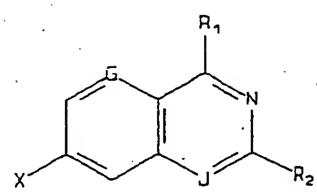

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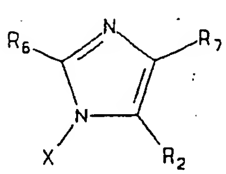

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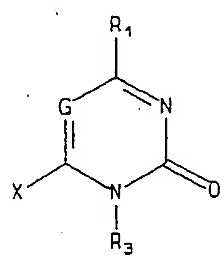


(11)

an oligonucleotide and other groups as described above for the group R_C. It is preferred that X have the general structure (4) or (5).

For the purposes of this invention, improving pharmacodynamic properties means improving oligonucleotide uptake, enhanced oligonucleotide resistance to degradation, and/or strengthened sequence-specific hybridization with RNA and improving pharmacokinetic properties means improved oligonucleotide uptake, distribution, metabolism or excretion. RNA cleaving moieties are chemical compounds or residues which are able to cleave an RNA strand in either a random or, preferably, a sequence-specific fashion.

Exemplary base moieties of the invention are any of the natural pyrimidinyl-1- or purinyl-9- bases including uracil, thymine, cytosine, adenine, guanine, 5-alkylcytosines such as 5-methylcytosine, hypoxanthine, 2-aminoadenine, and other modified bases as depicted in the formulas above. Exemplary sugars include ribofuranosyl, 2'-deoxyribofuranosyl, their corresponding five membered ring carbocyclic analogs as well as other modified sugars depicted in the formulas above. Particularly preferred modified sugars include 2'-fluoro and 2'-O-methyl-2'-deoxyribofuranosyl, i.e. 2'-fluoro and 2'-O-methyl-ß-D-erythro-pentofuranosyl.

Lower alkyl groups of the invention include but are not limited to C_1-C_{12} straight and branched chained, substituted or unsubstituted alkyls such as methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl,

ridines, phenazines, azidobenzenes, psoralens, porphyrins, cholesterols, and other "conjugate" groups.

Sugar derivatizing groups include, but are not limited to H, OH, alkyl, alkenyl, alkynyl, substituted alkyl, substituted alkenyl, substituted alkynyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, O-alkenyl, O-alkynyl, substituted O-alkyl, substituted O-alkenyl, substituted O-alkynyl, S-alkyl, S-alkenyl, S-alkynyl, substituted S-alkyl, substituted S-alkenyl, substituted S-alkynyl, SOMe, SO_2Me, ONO_2, NO_2, N_3, NH_2, NH-alkyl, NH-alkenyl, NH-alkynyl, substituted NH-alkyl, substituted NH-alkenyl, substituted NH-alkynyl, $OCH_2CH=CH_2$, $OCH=CH_2$, OCH_2CCH, OCCH, aralkyl, aralkenyl, aralkynyl, heteroaralkyl, heteroaralkenyl, heteroaralkynyl, heterocycloalkyl, poly-alkylamino, substituted silyl, an RNA cleaving moiety, a group which improves the pharmacodynamic properties of an oligonucleotide, or a group which improves the pharmacokinetic properties of an oligonucleotide.

Methods of synthesizing such modified nucleosides are set forth in copending applications for United States Letters Patent, assigned to the assignee of this invention, and entitled Compositions and Methods for Modulating RNA Activity, serial number 463,358, filed January 11, 1990; Sugar Modified Oligonucleotides That Detect And Modulate Gene Expression, serial number 566,977, filed August 13, 1990; and Compositions and Methods for Modulating RNA Activity, serial number US91/00243, filed January 11, 1991, the entire disclosures of which are incorporated herein by reference.

measured by using the UV spectrum to determine the formation and breakdown (melting) of hybridization. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently a reduction in UV absorption indicates a higher T_m. The higher the T_m, the greater the strength of the binding of the strands. Non Watson-Crick base pairing has a strong destabilizing effect on the T_m. Consequently, as close to optimal fidelity of base pairing as possible is desired to have optimal binding of an oligonucleotide to its targeted RNA.

Oligonucleotides of the invention can also be evaluated as to their resistance to the degradative ability of a variety of exonucleases and endonucleases. Oligonucleotides of the invention may be treated with nucleases and then analyzed, as for instance, by polyacrylamide gel electrophoresis (PAGE) followed by staining with a suitable stain such as Stains All™ (Sigma Chem. Co., St. Louis, MO). Degradation products may be quantitated using laser densitometry.

Fetal calf and human serum can be used to evaluated nucleolytic activity on oligonucleotides having substantially chirally pure intersugar linkages. For instance a oligonucleotide having substantially all-Rp intersugar linkages may be evaluated in this manner. Testing on combinations of 3′ or 5′ end capped (having one or several phosphate linkages per cap) molecules may be used to establish a combination that yields greatest nuclease stability. Capping can be effected

assayed via Northern blot analysis for catalytic cleavage of mRNA by endogenous RNase H. This allows for determination of the effects of chirality on mammalian RNAse H activity.

Oligonucleotides having substantially chirally pure intersugar linkages can also be evaluated for inhibition of gene expression in cell culture model systems. To determine if an oligonucleotide having substantially pure chirally pure intersugar linkages is more potent or a more specific inhibitor of gene expression, an oligonucleotide having substantially chirally pure intersugar linkages designed to target reporter genes may be synthesized and tested in cell culture models of gene expression. The use of the vector pSV2CAT has previously been described to measure antisense effects on gene expression; see Henthorn, P., Zervos, P., Raducha, M., Harris, H., and Kadesch, T., *Proc.Natl.Acad.Sci.USA*, 85:6342-6346 (1988). This vector contains the bacterial chloramphenicol acetyl transferase gene under regulatory controls of the SV40 promoter. Utilizing a 15-mer oligonucleotide having all-Rp intersugar linkages of a sequence complementary to the initiation of translation of the CAT mRNA, pSV2CAT may be transfected into HeLa cells and, following treatment of the cells for 48 hr with an oligonucleotide having all-Rp intersugar linkages, CAT activity may then be assayed in the cells. The activity of an oligonucleotide having substantially chirally pure intersugar linkages in inhibition of gene expression may then be compared directly with a chemically synthesized random

tubulin, papilloma virus (HPV), the ras oncogene and proto-oncogene, ICAM-1 (intercellular adhesion molecule-1) cytokine, and 5'-lipoxygenase. A targeted region for HSV includes GTC CGC GTC CAT GTC GGC (SEQ ID NO:1). A targeted region for HIV includes GCT CCC AGG CTC AGA TCT (SEQ ID NO:2). A targeted region for *Candida albicans* includes TGT CGA TAA TAT TAC CA (SEQ ID NO:3). A targeted region for human papillomavirus, e.g. virus types HPV-11 and HPV-18, includes TTG CTT CCA TCT TCC TCG TC (SEQ ID NO:4). A targeted region for ras includes TCC GTC ATC GCT CCT CAG GG (SEQ ID NO:5). A targeted region for ICAM-1 includes TGG GAG CCA TAG CGA GGC (SEQ ID NO:6) and the sequence CGA CTA TGC AAG TAC (SEQ ID NO:9) is a useful target sequence for 5-lipoxygenase. In each of the above sequences the individual nucleotide units of the oligonucleotides are listed in a 5' to 3' sense from left to right.

The phosphorothioate, methylphosphonate, phosphotriester or phosphoramidate oligonucleotides of the invention may be used in therapeutics, as diagnostics, and for research, as specified in the following copending applications for United States Letters Patent assigned to the assignee of this invention: Compositions and Methods for Detecting and Modulating RNA Activity, Serial No. 463,358, filed January 11, 1990; Antisense Oligonucleotide Inhibitors of Papilloma Virus, Serial No. 445,196 Filed 12/4/89; Oligonucleotide Therapies for Modulating the Effects of Herpesvirus, Serial No. 485,297, filed 2/26/90; Reagents and Methods for Modulating Gene Expression Through RNA Mimicry Serial No. 497,090, filed

by using the methodology of Ludwig and Eckstein; Ludwig, et al., *J. Org. Chem.* **1989**, *54*, 631-635. In this exemplary synthetic scheme, unprotected nucleosides can be reacted with 2-chloro-4H-1,3,2-benzodioxaphosphrin-4-one, which phosphitylates the 5'-hydroxyl group. Subsequent reaction with pyrophosphate yields cyclic triphosphate derivatives which are reactive to sulfur, yielding mixtures of Rp and Sp nucleoside 5'-O-(1-thiotriphosphates), i.e. alpha-thiotriphosphates. The products can be purified such as by using DEAE-Sephadex chromatography and identified with NMR spectroscopy by characteristic Rp or Sp chemical shifts.

As is shown in the examples below, pure Rp and Sp nucleosides 5'-O-(1-thiotriphosphates) diastereomers can be readily isolated on a preparative scale using, for example, reverse phase HPLC chromatography. Such HPLC isolated nucleotide diastereomers can be further characterized by analytical HPLC comparisons with commercial samples of such Rp and Sp nucleoside 5'-O-(1-thiotriphosphates) diastereomers.

Enzymatic synthesis of sequence specific natural oligonucleotides, i.e. natural phosphodiester oligonucleotides, can be effected by the use of an appropriate nuclease in the presence of a template and primer. In a like manner racemic mixtures of oligonucleotides having chirally mixed intersugar linkages can be synthesized. According to the teachings of the present invention, such enzymatic synthesis can also be expanded to include the synthesis of sequence specific oligonucleotides having substantially chirally pure

T7 DNA polymerase, modified T7 DNA polymerases such as the above referenced Sequenase, *E. coli* DNA polymerase, DNA poly Klenow fragment polymerase, M. luteus polymerase, T4 bacteriophage polymerase, modified T4 DNA polymerase, T7 RNA polymerase and *E. coli* RNA polymerase.

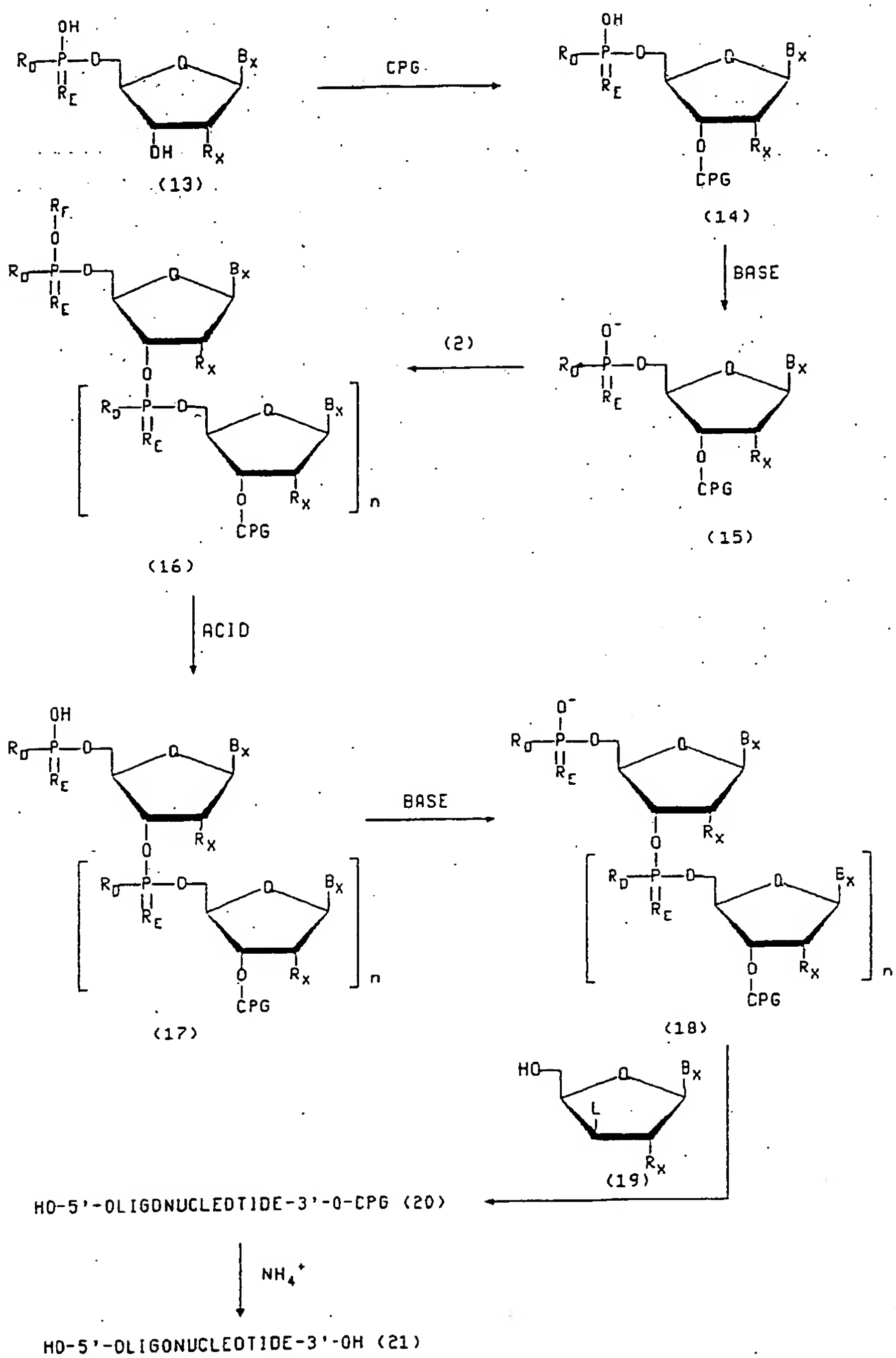
The enzymatic synthesis proceeds with inversion about the chiral center of the phosphorus atom. For example, the use of all-Sp alpha-thiotriphosphates yields substantially all Rp phosphorothioate oligonucleotides while use of all-Rp alpha-thiotriphosphates yields substantially all Sp phosphorothioate oligonucleotides. Alternatively oligonucleotides having chiral phosphate linkages such as phosphorothioate oligonucleotides may be synthesized from Sp-Rp racemic mixtures of nucleoside, such as 5'-O-(1-thiotriphosphates) utilizing metal ions in reaction solutions to promote preferential incorporation of one or the other of the chiral alpha-S-triphosphates. As noted above polymerase synthesis of such phosphorothioate oligonucleotide is accomplished with inversion about the chiral center of the precursor nucleoside alpha-S-triphosphate. While not wishing to be bound by theory, it is believed that optimization of an all-Rp configuration may be accomplished by addition of a (relative) high concentration of magnesium ion in the reaction buffer utilizing for instance an *E. coli* polymerase. In a like manner, again while we do not wish to be bound by theory, an all-Sp configuration might be obtained by utilizing a

a first 5′ end nucleoside from said primer. Additional nucleosides of said oligonucleotides of the present invention are those nucleoside added via enzymatic methods.

By selecting appropriate restriction nucleases in conjuction with selected primers, various 5′-terminal nucleosides of desired oligonucleotides are appropriately positioned at the 5′ end of an oligonucleotide. Thus, any endonuclease recognition site can be designed as long as the staggered cut results in one nucleoside from the primer being the first 5′ nucleoside of the newly synthesized sequence specific oligonucleotide of the invention. This results in the generation of different nucleosides on 5′ ends of enzymatically synthesized oligonucleotides of the invention.

Upon completion of enzymatic extension of said primer on an appropriate template of a desired sequence, oligonucleotides of the invention may be released from said primer by use of appropriate nuclease. For example, for incorporation of a guanosine nucleoside at the 5′ end of desired oligonucleotides, a primer having an CTGCAG sequence at its 3′ terminal end may be used. Use of a Pst 1 restriction nuclease then may cleave the A-G linkage. The guanosine nucleoside component of this A-G linkage may thus incorporated as a 5′ terminal nucleoside of desired oligonucleotides. Other restriction endonuclease include but are not limited to BamH1, Smal and HinD III restriction endonucleases.

SCHEME 1


CPG
(13)
(14)
BASE
(2)
(15)
(16)
ACID
BASE
(17)
(18)
(19)
HO-5'-OLIGONUCLEOTIDE-3'-O-CPG (20)
NH4+
HO-5'-OLIGONUCLEOTIDE-3'-OH (21)

second synthon to the first synthon to yield compound (16) wherein n = 1 and washing, the phosphate blocking group R_F is removed with an acid, yielding compound (17) wherein n = 1. Compound (17), which represents a new first synthon, is now treated with base to generate a further anionic, compound (18) with n = 1. Compound (18) is suitable for nucleophilic attack on a further unit of compound (2) (the second synthon) to form a new compound (16) wherein n = 2. In this further unit having compound (2), the B_X moiety may be the same or different from the B_X moiety of either of the nucleotides of compound (16) wherein n = 1, depending on the desired sequence.

Compound (16) wherein n = 2 is washed and then treated with acid to deblock the R_F blocking group, yielding a further new first synthon, compound (17) wherein n = 2. This new first synthon, is now ready to be further cycled by treatment with base to yield compound (18) wherein n = 2, which is now reacted with a further unit having compound (2) to yield a further unit of having structure (16) wherein n = 3. Again, B_X may be the same or different than previously B_X moieties. The cycle is repeated for as many times as necessary to introduced further nucleotides of the desired sequence via compound (2).

If it is desired to have the 5′ terminal end of the final oligonucleotide as a phosphate group, then the last compound (17) is appropriately removed from the CPG support. If it is desired to have the 5′ terminal end as a hydroxyl

ribofuranosyl sugar conformation) that is identical to natural or wild type oligonucleotides.

The second synthon carries a phosphate blocking group on its phosphorothioate, methylphosphonate, phosphotriester or phosphoramidate phosphorus group. After coupling of the second synthon to the first synthon, this phosphate blocking group is removed, generating a new first synthon having an anion at its 5′ phosphate suitable for nucleophilic attack on a further second synthon. Thus, after coupling of the first and second synthon, the newly joined first and second synthons now form a new first synthon. The oligonucleotide is elongated nucleotide by nucleotide via the nucleophilic attack of a phosphate anion at the 5′ end of the growing oligonucleotide chain on the leaving group at the 3′ position of the soon-to-be-added xylofuranosyl configured second synthon nucleotide.

It is presently preferred that the phosphate blocking group be a base stable, acid labile group. Such a phosphate blocking group maintains the phosphate moiety of the second synthon in a protected form that cannot react with the leaving group of the second synthon. This inhibits polymerization of the second synthon during the coupling reaction.

The nucleophilic coupling of the first and second synthons is a stereoselective coupling process that maintains the stereospecific configuration about the phosphorus atom of the first synthon. Thus the particular Sp or Rp diastereomeric configuration of a resolved phosphorothioate,

growing oligonucleotide chain, the inter-nucleotide linkage between nucleotides three and four is also an Rp diastereomeric linkage. If each added "second synthon" is also an Rp diastereomer, then the resulting oligonucleotide will contain only Rp inter-nucleotide linkages. If an oligonucleotide having Sp inter-nucleotide linkages is desired, then the first nucleotide and each of the added subsequent nucleotides are selected as Sp diastereomeric nucleotides.

The first synthon can be a first nucleotide or a growing oligonucleotide chain. If it is desired that each of the nucleotides of the oligonucleotide be ribofuranoside configured nucleotides, then the first nucleotide is selected as a ribofuranoside configured nucleotide. Each added second synthon, while added as a xylofuranoside configured nucleotide, after inversion is converted to a ribofuranoside configured nucleotide.

The 3′ position of the first nucleotide is either blocked if a solution reaction is practiced or is coupled to a solid state support if a solid state reaction (as for instance one utilizing a DNA synthesizer) is practiced. Each additional nucleotide of the oligonucleotide is then derived from a xylofuranosyl nucleotide, i.e. a second synthon. Because the first nucleotide of the oligonucleotide can be a "standard" ribofuranosyl nucleotide coupled via its 3′ hydroxyl to a solid state support, the standard solid state supports known in the art, such as controlled pore glass (CPG)

The second synthon is added either concurrently with the base or subsequent to it. After coupling, the growing oligonucleotide is washed with a solvent and then treated with a reagent to effect deblocking of the phosphate blocking group of the second synthon. If a preferred acid-labile blocking group is used to block the phosphate of the second synthon, deblocking is easily effected by treating the growing oligonucleotide on the solid state support with an appropriate acid.

Suitable acid-labile blocking groups for the phosphates of the second synthon include but are not limited to t-butyl, dimethoxytrityl (DMT) or tetrahydropyranyl groups. Suitable acids for deblocking the second synthon phosphate blocking group include but are not limited to acetic acid, trichloroacetic acid, and trifluoromethane sulfonic acid. Such acids are suitably soluble in solvents such as tetrahydrofuran, acetonitrile, dioxane, and the like.

Following treatment with an appropriate deblocking reagent to effect deblocking of the phosphate protecting group, the growing oligonucleotide is then washed with an appropriate solvent such as tetrahydrofuran, acetonitrile or dioxane. The oligonucleotide is now ready for the addition of a further nucleotide via treatment with base to generate an anion on the 5′ terminal phosphate followed by the addition of a further second synthon. Alternatively, the anion can be generated concurrently with addition of a further second synthon. Suitable leaving groups for inclusion at the 3′

pyrimidine base or the 3' position of the sugar and the 8 position of the purine base can be oxygen, sulfur or nitrogen.

Since a basic environment is created during coupling of the first synthon to the second synthon and an acidic environment (utilizing the preferred acid-labile phosphate blocking group) is created during deblocking of the phosphate blocking group from the nucleotide derived from the second synthon, if blocking groups are utilized on the base or sugar portions of the nucleotides such base or sugar blocking groups must be stable to both acidic and basis conditions. Suitable blocking groups for the heterocyclic base or the sugar are selected to be stable to these conditions. One type of blocking groups that can be used are acid\base stable, hydrogenolysis-sensitive blocking groups; that is, blocking groups which can be removed with molecular hydrogen but not with acid or base. A benzyl blocking group is such a suitable hydrogenolysis-sensitive blocking group.

Other heterocycle base or sugar blocking groups are those that require more pronounced acid or base treatment to de-block than may be experienced during the basic activation of the nucleophilic displacement reaction of the second synthon blocking group or the acidic removal of the phosphate blocking group. Two such blocking groups are the benzoyl and isobutyryl groups. Both of these require strong basic conditions for their removal. These basic conditions are more stringent than that required to generate the phosphate anion for the nucleophilic displacement reaction. This allows the

propyl, 2′-chloro, 2′-iodo, 2′-bromo, 2′-amino, 2′-azido, 2′-O-methyl, 2′-O-ethyl, 2′-O-propyl, 2′-O-nonyl, 2′-O-pentyl, 2′-O-benzyl, 2′-O-butyl, 2′-O-(propylphthalimide), 2′-S-methyl, 2′-S-ethyl, 2′-aminononyl, 2′-aralkyl, and 2′-alkylheterocyclo such as propylimidazoyl derivatives of the above 2′-deoxy-threo-pentofuranosyl nucleosides. Representatives of this group include but are not limited to 9-(ß-D-2′-deoxy-2′-fluoro-threo-pentofuranosyl)adenine, 9-(ß-D-2′-deoxy-2′-fluoro-threo-pentofuranosyl)guanine, 9-(ß-D-2′-deoxy-2-fluoro-threo-pentofuranosyl)hypoxanthine, 1-(ß-D-2′-deoxy-2′-fluoro-threo-pentofuranosyl)uracil, 1-(ß-D-2′-deoxy-2′-fluoro-threo-pentofuranosyl)cytosine, 1-(ß-D-2′-deoxy-2′-fluoro-threo-pentofuranosyl)thymine, 5-methyl-1-(ß-D-2′-deoxy-2′-fluoro-threo-pentofuranosyl)cytosine, 2-amino-9-(ß-D-2′-deoxy-2′-fluoro-threo-pentofuranosyl)adenine, 9-(ß-D-2′-deoxy-2′-methoxy-threo-pentofuranosyl)adenine, 9-(ß-D-2′-deoxy-2′-methoxy-threo-pentofuranosyl)guanine, 9-(ß-D-2′-deoxy-2-methoxy-threo-pentofuranosyl)hypoxanthine, 1-(ß-D-2′-deoxy-2′-methoxy-threo-pentofuranosyl)uracil, 1-(ß-D-2′-deoxy-2′-methoxy-threo-pentofuranosyl)cytosine, 1-(ß-D-2′-deoxy-2′-methoxy-threo-pentofuranosyl)thymine, 5-methyl-1-(ß-D-2′-deoxy-2′-methoxy-threo-pentofuranosyl)cytosine,2-amino-9-(ß-D-2′-deoxy-2′-methoxy-threo-pentofuranosyl)adenine, 9-(ß-D-2′-deoxy-2′-O-allyl-threo-pentofuranosyl)adenine, 9-(ß-D-2′-deoxy-2′-O-allyl-threo-pentofuranosyl)guanine, 9-(ß-D-2′-deoxy-2′-O-allyl-threo-pentofuranosyl)hypoxanthine, 1-(ß-D-2′-deoxy-2′-O-allyl-threo-pentofuranosyl)uracil, 1-(ß-D-2′-deoxy-

Another preferred group of nucleoside precursors for the second synthon include the carbocyclic nucleosides, i.e. nucleosides having a methylene group in place of the pentofuranosyl ring oxygen atom. Such carbocyclic compounds may exhibit increased stability towards chemical manipulation during activation of the xylo nucleosides for nucleophilic attack.

The xylo nucleoside or derivatized xylo nucleoside is reacted with a suitable phosphorylating agent to phosphorylate the second synthon precursor. Various phosphorylation reactions are known in the art such as those described in *Nucleotide Analogs*, by Karl Heinz Scheit, John Wiley & Sons, 1980, Chapter Four - Nucleotides with Modified Phosphate Groups and Chapter Six - Methods Of Phosphorylation; Conjugates Of Oligonucleotides and Modified Oligonucleotides: A Review Of Their Synthesis and Properties, Goodchild, J. (1990), *Bioconjugate Chemistry*, 1:165; and Antisense Oligonucleotides: A New Therapeutic Principle, Uhlmann, E. and Peyman, A. (1990), *Chemical Reviews*, 90:543.

Preferred phosphorylating agents include phosphoryl chlorides. Suitable phosphoryl chlorides include but are not limited to thiophosphoryl chloride, t-butoxyphosphoryl chloride, t-butoxy(methyl)phosphoryl chloride, t-butoxy-(methyl)thiophosphoryl chloride, t-butoxy(methoxy)phosphoryl chloride. Other phosphoryl chlorides may include t-butoxy(N-morpholino)phosphoryl chloride, t-butoxy(ethoxy-ethylamino)phosphoryl chloride, t-butoxy(methy-

triethylphosphate, Murray, A.W. and Atkinson, M.R. (1968), *Biochemistry*, 7:4023.

The appropriate phosphorylated xylo nucleotide is then activated for nucleophilic displacement at its 3′ position by reacting the 3′-hydroxyl group of the xylo compound with an appropriate anhydride, chloride, bromide, acyloxonium ion, or through an anhydro or cyclo nucleoside or the like to convert the 3′-hydroxyl group of the xylo nucleoside to an appropriate leaving group.

In a further method of synthesis, treatment of 2′,3′-anhydroadenosine with sodium ethylmercaptide gives 9-[3-deoxy-3-(ethylthio)-ß-D-xylofuranosyl]adenine. Treatment of this compound with a first synthon nucleophile may generate a terminal 2-ethylthio arabinofuranosyl nucleoside that could be desulfurized to yield the corresponding 2′-deoxynucleoside.

If during phosphorylation or conversion of the xylo 3′-hydroxyl to a 3′-activated leaving group stereospecific diastereomers are not obtained, after completion of the phosphorylation or conversion of the 3′-hydroxyl to an activated leaving group, the Rp and Sp diastereomers of these compounds will then be isolated by HPLC. This will yield pure diastereomers in a stereospecific form ready for use as the second synthons of Scheme 1.

Additional objects, advantages, and novel features of this invention will become apparent to those skilled in the art upon examination of the following examples thereof, which are not intended to be limiting.

EXAMPLE 3

N^6-Benzoyl-9-(2'-Deoxy-2'-fluoro-threo-pentofuranosyl)adenine

In a manner similar to Example 1, Method A, N^5-benzoyladenine is condensed with 1,3,5-tri-O-acetyl-2-deoxy-2-fluoro-D-threo-pentofuranoside to yield the title compound.

EXAMPLE 4

1-(2'-Deoxy-2'-methoxy-ß-D-xylofuranosyl)uridine

In a manner similar to Example 1, Method A, uracil is condensed with 1,3,5-tri-O-acetyl-2-deoxy-2-methoxy-D-threo-pentofuranoside to yield the title compound.

EXAMPLE 5

1-(2'-Deoxy-2'-O-allyl-ß-D-threo-pentofuranosyl)cytosine

In a manner similar to Example 1, Method A, cytosine is condensed with 1,3,5-tri-O-acetyl-2-deoxy-2-O-allyl-D-threo-pentofuranoside to yield the title compound.

EXAMPLE 6

Xyloguanosine

Method A

In a manner similar to Example 1, Method A, guanine is condensed with 1,2,3,5-tetra-O-acetyl-D-xylopentofuranoside to yield the title compound.

Method B

The chloromercury derivative of 2-acetamido-6-chloropurine is condensed with 2,3,5-tri-O-acetyl-ß-D-

pyrimidine intermediate that is aminated and ring closed to yield the carbocyclic analog of xylofuranosyladenine as per the procedure of Vincé, R. and Daluge, S. (1972), *J. Med. Chem.*, 15:171.

EXAMPLE 11

Carbocyclic Xyloinosine

5-Amino-6-chloro-pyrimidyl-4-one when treated with (±)-4α-amino-2α,3ß-dihydroxy-1α-cyclopentanemethanol will give a pyrimidine intermediate that is then aminated and ring closed to yield the carbocyclic analog of xylofuranosylinosine as per the procedure of Example 8.

EXAMPLE 12

O^2,3'-Cyclothymidine

Method A

3'-O-Mesylthymidine is treated with boiling water and the pH is adjusted to pH 4-5 according to the procedure of Miller, N. and Fox, J.J. (1964), *J. Org. Chem.*, 29:1771 to yield the title compound. This same compound can also prepared from 3'-deoxy-3'-iodothymidine by treatment with silver acetate in acetonitrile.

Method B

O^2,3'-Cyclothymidine and other 2'-deoxynucleosides are prepared by the treatment of the appropriate nucleoside with (2-chloro-1,1,3-trifluoroethyl)diethylamine in dimethylformamide according to the procedure of Kowollik, G., Gaertner, K., and Langen, P. (1969), *Tetrahedron Lett.*, 3863.

EXAMPLE 15

N^6,5′-Cyclothymidine

5′-O-Trityl-3′-O-mesylthymidine is treated with sodium azide to yield N^6,5′-cyclothymidine as one of the products. 5′-O-trityl-3′-O-mesylthymidine is also cyclizable to O^2,3′-cyclothymidine.

EXAMPLE 16

8,3′-Cycloadenosine

The anhydro ring from the 3′ position of the sugar to the 8 position of the purine ring is formed by treatment of 5′-O-acetyl-8-bromo-2′ (or 3′)-O-p-toluenesulfonyladenosine with thiourea to yield the 8,3′-thiocyclonucleoside (as well as the corresponding 8,2′) product as per the procedure of Ikehara, M. and Kaneko, M. (1970), *Tetrahedron*, 26:4251.

EXAMPLE 17

8,3′-Cycloguanosine

The title compound is prepared as per Example 16 utilizing 8-bromoguanosine. Both this compound and the compound of Example 16 can be oxidized to their corresponding sulfoxides via tert-butyl hypochlorite in methanol or treated with chlorine in methanolic hydrogen chloride to yield the 3′-sulfo-8-chloro analog in a procedure analogous with that of Mizuno, Y., Kaneko, O., and Oikawa, Y. (1974), *J. Org. Chem.*, 39:1440.

EXAMPLE 21

9-(3'-Chloro-3'-deoxy-ß-D-xylofuranosyl)hypoxanthine

5'-O-Acetylinosine is treated with triphenylphosphine and carbon tetrachloride to yield the title compound according to the procedure of Haga, K., Yoshikawa, M., and Kato, T. (1970), *Bull. Chem. Soc. Jpn.*, 43:3992.

EXAMPLE 22

9-(2-O-Acetyl-3-chloro-3-deoxy-5-O-pivaloyl-ß-D-xylofuranosyl)-6-pivalamidopurine

The title compound is prepared via an intermediate 2',3'-O-acyloxonium ion utilized to introduce a halogen atom at the 3' position and convert the ribo configuration of a nucleoside into the corresponding 3'-halo-3'-deoxy xylo nucleoside. The acyloxonium ion is generated *in situ* by treatment of 2',3'-O-methoxyethylidineadenosine with pivaloyl chloride in hot pyridine. Attack by chloride gives the title compound. Hypoxanthine and guanine nucleoside react in a similar manner. Sodium iodide will be used to generate the corresponding 3'-iodides according to the procedure of Robins, M.J., Fouron, Y., and Mengel, R. (1974), *J. Org. Chem.*, 39:1564.

EXAMPLE 26

1-(ß-D-2'-Deoxy-2'-fluoro-threo-pentofuranosyl)cytosine

In a manner similar to Example 3, cytosine is condensed with 1,3,5,-tri-O-acetyl-2-deoxy-2-fluoro-D-threo-pentofuranoside to yield the title compound.

EXAMPLE 27

O^2,3'-Cyclo-2'-deoxycytidine

The title compound is prepared by heating the 3'-O-sulfamate as per the procedure of Schuman, D., Robins, M.J., and Robins, R.K. (1970), *J. Am. Chem. Soc.*, 92:3434.

EXAMPLE 28

Sp and Rp Xyloadenosine 5'-Monophosphate

N^6-Benzoyl-xyloadenosine is phosphorylated with phosphoryl chloride in pyridine and acetonitrile at 0°C. The reaction will be quenched with ice water, rendered basic and added to an activated charcoal column. After elution with ethanol/water/concentrated ammonium hydroxide the solvent is evaporated to dryness and the residue dissolved in water and passed through an ion exchange column. The benzoyl blocking group is removed in concentrated ammonium hydroxide followed by separation of the diastereomers by HPLC to yield the title compound.

EXAMPLE 32

Sp and Rp 9-(ß-D-2'-Deoxy-2'-fluoro-threo-pentofuranosyl)guanine 5'-t-butoxy(methyl)phosphonate

9-(ß-D-2'-Deoxy-2'-fluoro-threo-pentofuranosyl)guanine will be phosphorylated and purified as per the procedure of Example 29 to give the diastereomers of the title compound.

EXAMPLE 33

Sp and Rp Xylofuranosyluracil 5'-t-butoxyphosphorothioate

Xylofuranosyluracil will be phosphorothioated with t-butoxythiophosphorylchloride in triethylphosphate utilizing the method of Murray, A.W. and Atkinson, M.R. (1968), *Biochemistry*, 7:4023. The diastereomers of the title compound are separated by HPLC.

EXAMPLE 34

Sp and Rp 9-(2'-Deoxy-2'-methyl-ß-D-threo-pentofuranosyl)guanine 5'-Methylphosphonate

9-(2'-Deoxy-2'-methyl-ß-D-threo-pentofuranosyl)guanine will be alkylphosphonated utilizing the procedure of Holy, A. (1967), *Coll. Czech. Chem. Commun.*, 32:3713. The racemic phosphorylation product is separated into its Sp and Rp diastereomers using HPLC chromatography to yield the title compound.

EXAMPLE 37

Sp and Rp 9-(2'-Deoxy-2'-methoxy-ß-D-threo-pentofuranosyl)-uracil 5'-Phosphate

9-(2'-Deoxy-2'-methoxy-ß-D-threo-pentofuranosyl)uracil will be phosphorylated according to the procedure of Example 28 to yield the racemic title compound. The diastereomers are separated by HPLC.

EXAMPLE 38

Sp and Rp 3-Deaza-9-(xylofuranosyl)guanine 5'-Phosphate

3-Deaza-9-(xylofuranosyl)guanine will be phosphorylated according to the procedure of Example 28 to yield the racemic title compound. The diastereomers are separated by HPLC.

EXAMPLE 39

Sp and Rp Xyloguanosine 5'-Phosphorothioate

Xyloguanosine will be phosphorothioated with thiophosphoryl chloride according to the procedure of Example 28 to yield the racemic title compound. The diastereomers are separated by HPLC.

EXAMPLE 40

Sp and Rp Carbocyclic Xyloadenosine 5'-Phosphate

In a like manner to Example 28, carbocyclic xyloadenosine will be treated with phosphoryl chloride to yield the racemic title compound. The diastereomers will be separated by HPLC.

EXAMPLE 43

9-(3'-Deoxy-3'-tosyl-2'-deoxy-2'-methoxy-ß-D-*threo*-pentofuranosyl)uracil 5'-Rp t-Butoxy(methyl)phosphonate

9-(2'-Deoxy-2'-methoxy-ß-D-*threo*-pentofuranosyl)uridine 5'-Rp t-butoxy(methyl)phosphonate will be treated with p-toluenesulfonylchloride in pyridine according to the procedure of Example 42 to yield the title compound.

EXAMPLE 44

9-(3'-Deoxy-3'-tosyl-2'-deoxy-2'-fluoro-ß-D-*threo*-pentofuranosyl)uracil 5'-Rp t-Butoxy(methyl)phosphonate

9-(2'-Deoxy-2'-fluoro-ß-D-*threo*-pentofuranosyl)uridine 5'-Rp t-butoxy(methyl)phosphonate will be treated with p-toluenesulfonylchloride in pyridine according to the procedure of Example 42 to yield the title compound.

EXAMPLE 45

9-(3'-Deoxy-3'-tosyl-2'-deoxy-2'-fluoro-ß-D-*threo*-pentofuranosyl)cytosine 5'-Rp t-Butoxy(methyl)phosphonate

9-(2'-Deoxy-2'-fluoro-ß-D-*threo*-pentofuranosyl)cytosine 5'-Rp t-butoxy(methyl)phosphonate will be treated with p-toluenesulfonylchloride in pyridine according to the procedure of Example 42 to yield the title compound.

EXAMPLE 49

Carbocyclic3'-Deoxy-3'-trifluoromethanesulfonylxyloadenosine 5'- Phosphate

In a like manner to Example 48, carbocyclic xyloadenosine 5'-Rp phosphate will be treated with trifluoromethane sulfonic acid to yield the title compound.

EXAMPLE 50

S^2,3'-Cyclo-2-thiothymidine

S^2,3'-Cyclo-2-thiothymidine is prepared from 3'-O-mesyl-O^2,5'-cyclothymidine via methanolysis followed by sulfhydryl ion attack. The S^2,3'-cyclo linkage is then opened up with base to yield 2',3'-dideoxy-3'-mercapto-1-(ß-D-xylofuranosyl)thymidine, Wempen, I. and Fox, J.J. (1969), *J. Org. Chem.*, 34:1020. The 3' position will then be activated to nucleophilic attack via an active leaving group such as conversion of the mercapto to a tosyl leaving group. In a like manner S^2,3'-Cyclo-2-thiouridine prepared from 2-thio-uridine by the method of Doerr, I.L. and Fox, J.J. (1967), *J. Am. Chem.*, 89:1760, can be ring opened and then derivatized with an activated leaving group such as a tosylate.

EXAMPLE 51

Synthesis of 2'-Deoxy-2'-fluoro substituted CGA CTA TGC AAC TAC Rp Methylphosphonate Linked Oligonucleotide

1-(2'-Fluoro-2'-deoxy-ß-D-ribofuranosyl)cytosine 5'-Rp methylphosphonate will be attached via its 3' hydroxyl to CPG

xylofuranosyl nucleoside. The oligonucleotide is concurrently deblocked and removed from the CPG beads by treatment with concentrate ammonium hydroxide.

EXAMPLE 52

Isolation of All-Sp or All Rp 5'-O-(1-thiotriphosphate) Nucleoside

5'-O-(1-thiotriphosphate) deoxynucleosides and ribonucleosides are isolated using C-18 reverse phase high performance liquid chromatography (HPLC) using columns packed with ODS Hypersil (Shandon Southern, Runcon, UK) and eluted with an isocratic mixture of solvent A (30 mM potassium phosphate containing 5 mM tetrabutylammonium ion, pH 7.0) and solvent B (5 mM tetrabutylammonuium hydroxide in methanol). Alternatively, effective separation is achieved using 100 mM triethylammonium bicarbonate, pH 7.5, containing a linear gradient of acetonitrile from 0% to 15% over 20 minutes.

To establish the purity of such HPLC separated enantiomers the HPLC separated Sp and Rp deoxynucleotide enantiomers are compared to commercially available deoxynucleoside 5'-O-(1-thiotriphosphates) available from E.I. Dupont, Wilmington, DE.

E. coli DNA polymerase I shows the dinucleoside to be of the Rp configuration.

EXAMPLE 54

Synthesis of Phosphorothioate CGA CTA TGC AAG TAC (SEQ ID NO:9) Oligonucleotide Having Substantially Pure Rp Intersugar Linkages

A large scale enzymatic synthesis of sequence specific all-Rp phosphorothioate oligonucleotide was effected utilizing a 55 mer natural phosphodiester template and a 41 mer natural phosphodiester primer. The template sequence was: GTA CTT GCA TAG TCG ATC GGA AAA TAG GGT TCT CAT CTC CCG GGA TTT GGT TGA G (SEQ ID NO: 7). The primer sequence was: CTC AAC CAA ATC CCG GGA GAT GAG AAC CCT ATT TTC CGA TC (SEQ ID NO:8). The template was selected to have a sequence complementary to a desired specific CGA CTA TGC AAG TAC (SEQ ID NO:9) sequence. A Sequenase™ buffer (U.S. Biochemicals Corp., Cleveland, OH) diluted from 5X down to 1X was used. The template and primer, both at concentrations of 20nM are added to 40 µL of this buffer. The template and primer were hybridized at 95 °C for 5 minutes and cooled to room temperature. After cooling the buffer was adjusted to 7 mM DTT. 20 µL 1:8 diluted Sequenase™ enzyme and 320 µM each of Sp GTPαS, CTPαS, ATPαS and TTPαS are then added. The reaction solution was adjusted to 140 µL with H_2O. It was incubated at 37 °C for 18 hours. The reaction solution was extracted 2X with a like volume of phenol in a standard manner and precipated in a standard manner by

EXAMPLE 55

Synthesis of Phosphorothioate Oligonucleotides Having a Racemic Mixture of Intersugar Linkages Using Automated DNA Synthesis.

Oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using hydrogenphosphonate chemistry in a standard manner. See Agrawal, S., Goodchild, J., Civeria, M.P., Thornton, A.H., Sarin, P.S., and Zamecnik, P.C. (1988) *Proc. Natl. Acad. Sci. USA,* 85:7079-7083. After the final coupling step, the phosphorothioate linkages are generated by oxidizing the bound oligomer with sulfur in carbon disulfide/triethylamine/pyridine. After sulfur oxidation, standard deblocking procedures with ammonium hydroxide are used to release the oligonucleotides from the support and remove base blocking groups. The phosphorothioate oligonucleotides are purified by oligonucleotide purification column (OPC; ABI, Foster City, CA) chromatography and HPLC, using a Beckman System Gold HPLC. The HPLC-purified oligonucleotides are then precipitated with ethanol and assessed for final purity by gel electrophoresis on 20% acrylamide/7 M urea or by analytical HPLC. The authenticity of the oligonucleotide sequence was assessed by oxidation with iodine in pyridine/water and standard sequencing methods. These oligonucleotides contain a mixture of all possible combinations of Rp and Sp isomers at each phosphorous linkage.

products were purified on a 20% polyacrylamide/8M urea gel and sequenced by standard procedures.

EXAMPLE 57

Thermal Denaturation

Oligonucleotides (either phosphorothioate oligonucleotides of the invention or otherwise) were incubated with either the complementary DNA or RNA oligonucleotides at a standard concentration of 4 μM for each oligonucleotide in 100 mM ionic strength buffer (89.8 mM NaCl, 10 mM Na-phosphate, pH 7.0, 0.2 mM EDTA). Samples were heated to 90 °C and the initial absorbance taken using a Guilford Response II spectrophotometer (Corning). Samples were then slowly cooled to 15°C and the change in absorbance at 260 nm monitored during the heat denaturation procedure. The temperature was elevated 1 degree/absorbance reading and the denaturation profile analyzed by taking the first derivative of the melting curve. Data was also analyzed using a two-state linear regression analysis to determine the T_m and delta G. The results of these tests are shown in Table 1.

[^{35}S] in a pyridine/carbon disulfide mixture. The resulting radiolabeled phosphorothioate oligonucleotide can be purified by OPC chromatography and HPLC. Target mRNA are applied to nitrocellulose filters and baked at 80°C for 2 hours, blocked and then hybridized with the radiolabeled phosphorothioate oligonucleotide. Binding stringency is assessed by quantitating radiolabeled oligonucleotide eluted from the filters after increases in temperature or increases in the ionic strength of an eluting buffer, as for instance, Tris NaCl buffer. Eluted oligonucleotides are also assessed for their mobility in an anion exchange HPLC protocol isocratically utilizing phosphate buffer. Results are compared to the mobility of standard oligonucleotides prepared having racemic mixtures of intersugar linkages.

EXAMPLE 59

Nuclease Digestion

Determination of the rate of nuclease degradation of the phosphorothioate oligonucleotides in media containing 10% fetal calf serum (FCS) was carried out in Dulbecco's Modified Essential Medium (DMEM) containing 10% heat inactivated FCS. Heat inactivation of the FCS was carried out at 55 °C for 1 hour prior to addition to media. Oligonucleotides having racemic and chirally pure intersugar linkages were separately tested for resistance to nuclease digestion. 66μg/ml of each oligonucleotide were separately added to medium and incubated at 37 °C, at the time intervals indicated in Table 2. 15 μl aliquots were removed and added to 15 μl of 9 M urea in 0.1 M Tris-HCl (pH 8.3), 0.1 M boric acid and 2 mM EDTA. Aliquots were mixed by vortex and stored at −20°C. Polyacrylamide gel electrophoresis (PAGE) analysis was on 20% polyacrylamide/7 M urea slab gels. Following electrophoresis, gels were stained using "Stains All" (Sigma Chem. Co., St. Louis, MO). Following de-staining, gels were analyzed via laser densitometry using an UltraScan XL device (Pharmacia LKB

RNase H hybridization buffer for 30 minutes at 60 °C. Samples were slowly cooled to room temperature and then adjusted to 3.7 mg/ml BSA, 20 units *E. coli* RNase H (Promega), 142 mM DTT, 150 mM KCl, and 3 mM $MgCl_2$. Samples were incubated for 30 minutes at 37°C. Samples were then phenol extracted, ethanol precipitated, and analyzed by electrophoresis on 1.2% agarose gels following ethidium bromide staining. Markers were run on gels concurrently with the samples to determine approximate length of RNA samples.

EXAMPLE 61

A patient suffering from psoriasis is treated with 10μg/kg body weight of oligonucleotide sythesized according to the method of Example 3, incorporated in a cream. Daily application of the cream continues until the condition is relieved.

EXAMPLE 62

A patient infected with human papillomavirus HPV-11 is treated with oligonucleotide synthesized according to Example 3, having the sequence TTG CTT CCA TCT TCC TCG TC (SEQ ID NO: 4). 1000 μg/kg body weight of oligonucleotide is incorporated into a pharmaceutically acceptable carrier and administered by a single intravascular injection, repeated as necessary until the infection is resolved.

EXAMPLE 63

A patient infected with *Candida Albicans* is treated with oligonucleotide synthesized according to Example 3, having the sequence TGT CGA TAA TAT TAC CA (SEQ ID NO:3). 100 μg/kg body weight doses of oligonucleotide are administered orally in a pharmaceutically acceptable carrier every six hours for one week or until the infection is abated.

(A) TELEPHONE: 215-368-3100
(B) TELEFAX: 215-568-3439

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCCGCGTCC ATGTCGGC 18

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTCCCAGGC TCAGATCT 18

(2) INFORMATION FOR SEQ ID NO:3:

(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCCGTCATCG CTCCTCAGGG 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGGAGCCAT AGCGAGGC 18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGACTATGCA AGTAC 15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GACTATGCAA GTAC 14

Q is O or CH_2;

R_D is O, S, methyl, O-alkyl, S-alkyl, amino or substituted amino;

R_E is O or S;

R_F is H or a labile blocking group;

R_X is H, OH, or a sugar derivatizing group;

B_X is a naturally occurring or synthetic nucleoside base or blocked nucleoside base;

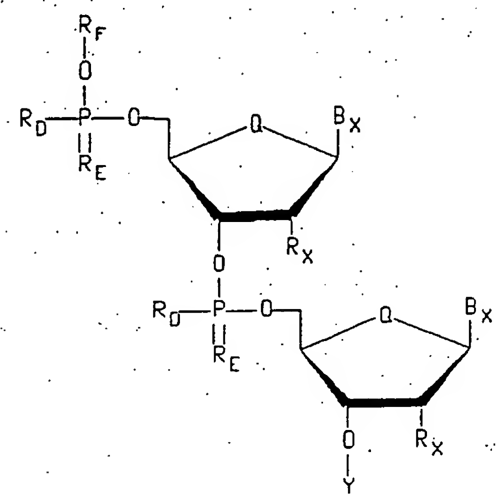
L is a leaving group or together L and Bx are a 2-3′ or 6-3′ pyrimidine or 8-3′ purine cyclo-nucleoside; and

Y is a stable blocking group, a solid state support, a nucleotide on a solid state support, or an oligonucleotide on a solid state support.

2. The method of claim 1 wherein L is selected from the group consisting of halogen, alkylsulfonyl, substituted alkylsulfonyl, arylsulfonyl, substituted arylsulfonyl, hetercyclcosulfonyl or trichloroacetimidate

3. The method of claim 1 wherein L is selected from the group consisting of chloro, fluoro, bromo, iodo, p-(2,4-dinitroanilino)benzenesulfonyl, benzenesulfonyl, methylsulfonyl (mesylate), p-methylbenzenesulfonyl (tosylate), p-bromobenzenesulfonyl, trifluoromethylsulfonyl (triflate),

9. The method of claim 1 wherein the new first synthon has the structure:



10. The method of claim 1 further comprising repeating said steps (b), (c), and (d) a plurality of times.

11. The method of claim 1 further comprising contacting said first synthon with said base in a solvent selected from the group consisting of acetonitrile, tetrahydrofuran and dioxane.

12. The method of claim 1 wherein said reagent used to remove blocking group R_F is an acid selected from the group consisting of trichloroacetic acid, acetic acid or trifluoromethane sulfonic acid.

13. The product of the process of claim 1.

cooling said template and primer mixture prior to the addition of the nucleoside triphosphate.

18. The method of claim 14 wherein said 5'-O-triphosphate is 5'-O-(thiotriphosphate).

19. The method of claim 18 wherein said 5'-O-thiotriphosphate is 2'-deoxyribonucleoside 5'-O-thiotriphosphates.

20. The method of claim 18 wherein said 5'-O-thiotriphosphates is ribonucleoside 5'-O-thiotriphosphates.

21. The method of claim 14 wherein said polymerase is T7 DNA polymerase, modified T7 DNA polymerase I, T7 RNA polymerase, T4 bacteriophage polymerase, modified T4 DNA polymerase, *M. luteus* polymerase, DNA poly Klenow fragment polymerase, *E. coli* RNA polymerase or *E. coli* DNA polymerase.

22. The method of claim 14 wherein dissassociation from the primer is accomplished by means of Pst 1 restriction endonuclease, BamH1 restriction endonuclease, Smal restriction endonuclease or HinD III restriction endonuclease.

23. The method of claim 14 wherein the dissassociation is achieved by DNAse I digestion.

28. The method of claim 26 wherein said metal ion is manganese.

29. The method of claim 26 wherein said template comprises a sequence of a target molecule.

30. The method of claim 26 wherein said primer has a restriction site located thereon.

31. The method of claim 26 further comprising the steps of:

prehybridizing said template and said primer by heating said template and said primer together; and

cooling said template and primer mixture prior to the addition of said triphosphates.

32. The method of claim 26 wherein said nucleoside 5'-O-triphosphate is 5'-O-(1-thiotriphosphate).

33. The method of claim 32 wherein said nucleoside 5'-O-(1-thiotriphosphate) is 2'-deoxyribonucleoside 5'-O-(1-thiotriphosphate).

34. The method of claim 32 wherein said nucleoside 5'-O-(1-thiotriphosphate) is ribonucleoside 5'-O-(1-thiotriphosphate).

41. The oligonucleotide of claim 40 wherein said phosphate linkages are selected from the group consisting of chiral Sp phosphorothioate, chiral Rp phosphorothioate, chiral Sp alkylphosphonate, chiral Rp alkylphosphonate, chiral Sp phosphoamidate, chiral Rp phosphoamidate, chiral Sp chiral phosphotriester or chiral Rp phosphotriester.

42. An oligonucleotide comprising a plurality of nucleoside units linked together via phosphate linkages, wherein:

at least one of the nucleoside units is a non-naturally occurring nucleoside unit; and

at least two of the nucleoside units are linked via chiral phosphate linkages.

43. The oligonucleotide of claim 42 wherein said chiral phosphate linkages are selected from the group consisting of chiral Sp phosphorothioate, chiral Rp phosphorothioate, chiral Sp alkylphosphonate, chiral Rp alkylphosphonate, chiral Sp phosphoamidate, chiral Rp phosphoamidate, chiral Sp chiral phosphotriester or chiral Rp phosphotriester.

44. The oligonucleotide of claim 42 wherein each of the phosphate linkages is a chiral phosphate linkage.

45. The oligonucleotide of claim 42 wherein:

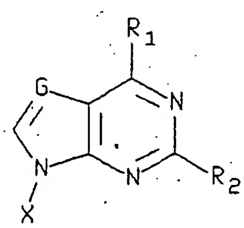
R_G is H, alkyl, substituted alkyl, an RNA cleaving moiety, a group which improves the pharmacokinetic properties of an oligonucleotide, or a group which improves the pharmacodynamic properties of an oligonucleotide; and

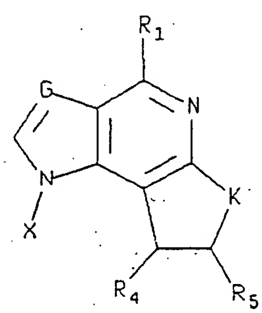
B_X is a naturally occurring or synthetic nucleoside base.

46. The oligonucleotide of claim 45 wherein B_X is a pyrimidinyl-1 or purinyl-9 moiety.

47. The oligonucleotide of claim 45 wherein B_X is adenine, guanine, hypoxanthine, uracil, thymine, cytosine, 2-aminoadenine or 5-methylcytosine.

48. The oligonucleotide of claim 42 wherein at least one of the modified nucleotides has one of the structures:



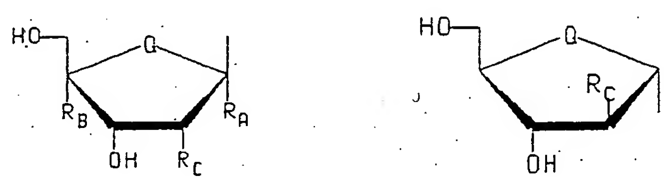


a group which improves the pharmacokinetic properties of an oligonucleotide, or a group which improves the pharmacodynamic properties of an oligonucleotide;

R_6 and R_7 are, independently, H, OH, NH_2, SH, halogen, $CONH_2$, $C(NH)NH_2$, C(O)O-alkyl, $CSNH_2$, CN, C(NH)NHOH, alkyl, substituted alkyl, substituted amino, an RNA cleaving moiety, a group which improves the pharmacokinetic properties of an oligonucleotide, or a group which improves the pharmacodynamic properties of an oligonucleotide; and

X is a sugar or a sugar substituted with at least one substituent comprising an RNA cleaving moiety, a group which improves the pharmacodynamic properties of an oligonucleotide, or a group which improves the pharmacokinetic properties of an oligonucleotide.

49. The oligonucleotide of claim 48 wherein X has one of the structures:



wherein:

Q is O or CHR_G;

R_A and R_B are H, alkyl, substituted alkyl, an RNA cleaving moiety, a group which improves the pharmacokinetic

51. An oligonucleotide comprising at least 10 nucleoside units linked together by all Sp phosphorothioate linkages or by all Rp phosphorothioate linkages.

52. An oligonucleotide comprising a plurality of nucleoside units linked together by all Sp phosphotriester linkages or by all Rp phosphotriester linkages.

53. The oligonucleotide of claim 52 wherein said nucleoside units are linked together in a sequence that is antisense to an RNA or DNA sequence.

54. An oligonucleotide comprising a plurality of linked of nucleoside units linked together by all Sp phosphoramidate linkages or by all Rp phosphoramidate linkages.

55. The oligonucleotide of claim 54 wherein said nucleoside units are linked together in a sequence that is antisense to an RNA or DNA sequence.

56. An oligonucleotide comprising at least 10 nucleoside units linked together by all Sp alkylphosphonate linkages or by all Rp alkylphosphonate linkages.

57. The oligonucleotide of claim 56 wherein said alkylphosphonate linkages are methylphosphonate linkages.

61. The compound of claim 59 wherein L is selected from the group consisting of chloro, fluoro, bromo, iodo, p-(2,4-dinitroanilino)benzenesulfonyl, benzenesulfonyl, methylsulfonyl (mesylate), p-methylbenzenesulfonyl (tosylate), p-bromobenzenesulfonyl, trifluoromethylsulfonyl (triflate), trichloroacetimidate, acyloxy, 2,2,2-trifluoroethanesulfonyl, imidazolesulfonyl and 2,4,6-trichlorophenyl.

62. The compound of claim 59 wherein R_X is H, OH, alkyl, alkenyl, alkynyl, substituted alkyl, alkenyl, alkynyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, O-alkenyl, O-alkynyl, substituted O-alkyl, substituted O-alkenyl, substituted O-alkynyl, S-alkyl, S-alkenyl, S-alkynyl, substituted S-alkyl, substitute S-alkenyl, substituted S-alkynyl, SOMe, SO_2Me, ONO_2, NO_2, N_3, NH_2, NH-alkyl, NH-alkenyl, NH-alkynyl, substituted NH-alkyl, substituted NH-alkenyl, substituted NH-alkynyl, $OCH_2CH=CH_2$, $OCH=CH_2$, OCH_2CCH, OCCH, aralkyl, aralkenyl, aralkynyl, heteroaralkyl, heteroaralkenyl, heteroaralkynyl, heterocycloalkyl, poly(alkylamino), substituted silyl, an RNA cleaving moiety, a group for improving the pharmacodynamic properties of an oligonucleotide or a group for improving the pharmacokinetic properties of an oligonucleotide.

63. The compound of claim 59 wherein R_F is selected from the group consisting of H, t-butyl, dimethoxytrityl or tetrahydropyranyl.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08797

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C 12 P 19/34; C 07 K 15/00; C 07 H 21/00
US CL :435/89, 91; 536/27, 28, 29; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Cohen, "Oligonucleotides," published 1989 by CRC Press, Inc. (Florida), see pages 7-116, 137-210.	1-69
Y	The Journal of Biological Chemistry, Volume 257, No 13, issued 10 July 1982, A. Gupta et al., "Template-Primer dependent Turnover of (Sp) - dATPaS by T4 DNA polymerase", see pages 7689-7692.	1-68
Y	The Journal of Biological Chemistry, Vol. 13, issued 10 July 1982, P.J. Romaniuk et al., "A Study of the Mechanism of T4 DNA Poymerase with Diastereomeric Phosphorothioate Analogues of Deoxyadenosine Triphosphate," see pages 7684-7688.	1-68

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 JANUARY 1993	15 JAN 1993

Name and mailing address of the ISA/	Authorized officer
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